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INSIGHTS FROM MODEL SYSTEMS
Genetic Causes of Female Infertility: Targeted Mutagenesis in Mice

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Introduction

Infertility, defined as the inability to conceive after 1 year of unprotected intercourse, affects 1 in 10 couples in the United States (Chandra and Stephen 1998). The etiologies of infertility are diverse. Although many causes, including inflammatory tubal disease, ovulatory dysfunction, azoospermia, and immotile cilia syndrome, are well established, the role of other factors, such as endometriosis, uterine fibroids, and varioceles, is surrounded by intense controversy. During initial clinical appraisals, each partner must be thoroughly evaluated, both because male and female factors contribute roughly equally to the etiology and because more than one cause of infertility may be present. Despite extensive diagnostic testing, 10% of infertility remains unexplained. These patients may suffer from subtle defects not identified by routine evaluation, or they may represent novel causes of infertility.

It seems likely that a subgroup of couples with unexplained infertility will have intrinsic gamete abnormalities preventing normal sperm-oocyte interaction and fertilization. This presumption is supported by the reduced fertility or complete failure of fertilization during in vitro procedures. Both the maturation of human gametes and the events surrounding in vivo fertilization are inaccessible to study by current technologies. However, recent advances in molecular biology and genetics have provided investigators with the tools to better understand mammalian fertilization. By means of targeted mutagenesis in embryonic stem cells and transgenesis, it is possible to create mouse lines lacking specific gene products and to analyze the phenotype. Although a significant number of mutant mice created by embryonic–stem-cell technology have hypofertility or infertility (Nishimori and Matzuk 1996), this review will focus only on a subset—those that affect the development of the female gamete and its surrounding egg coat, the zona pellucida (fig. 1).

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Folliculogenesis

Unlike males, who produce new sperm continuously throughout their lives, each female has her lifetime complement of germ cells at birth. These "resting" oocytes and their surrounding layer of squamous granulosa cells form the primordial follicle. Cohorts of these follicles are induced to enter a growth phase, which culminates (after some weeks, depending on species) in meiotic maturation and ovulation. Concomitant with the onset of granulosa-cell proliferation, the oocyte initiates its own growth while remaining arrested in the prophase of the first meiotic division. The nuclear and cytoplasmic volumes enlarge as the diameter of the oocyte increases. During this initial growth, the zona pellucida is first observed as extracellular patches, which later coalesce into a uniform matrix surrounding the oocyte.

In contrast to early folliculogenesis, follicular development to the preovulatory stage is gonadotropin dependent and results in the formation of a central cavity, the antrum. At approximately the same time as the antrum forms, the oocyte stops growing and acquires competence to resume meiosis. However, interactions with follicle cells maintain the oocyte in meiotic arrest until shortly before ovulation. The surrounding zona pellucida matrix (7 μm thick in the mouse; 15 μm thick in humans) physically separates the oocyte and granulosa cells, but close associations are maintained throughout follicular development, via paracrine factors and cellular processes that penetrate the zona to form gap junctions. In the late stages of folliculogenesis, a surge of luteinizing hormone (LH) triggers disappearance of the gap junctions and releases the oocyte from meiotic arrest, for completion of the first meiotic division. The cumulus oophorus, in whose center lies a mature egg ready for fertilization, expands and is released during ovulation.

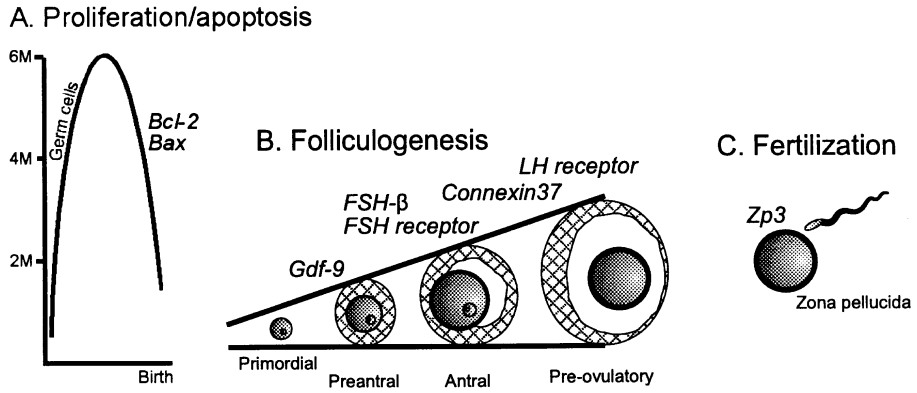


Figure 1 Genetic mutations affecting female fertility. During human gestation, millions of female germ cells are formed, but only 200–400,000 persist at menarche, and <500 are ovulated prior to menopause. Throughout a women's reproductive life, multiple follicles enter a growth phase as a cohort, but only one becomes the dominant Graafian follicle and available for fertilization. *A,* Germ-cell proliferation and apoptosis during gestation. *B,* Folliculogenesis during which oocytes grow and complete the first meiotic division while interacting with the surrounding follicular cells. *C,* Sperm-egg interactions at fertilization.

Proliferation and Atresia

The vast majority of growing and nongrowing follicles are lost by atresia. In humans, this process is particularly rapid during the neonatal period, when the total oocyte endowment declines from 6 million at 20 wk gestation to 1–2 million at birth (Erickson 1986). As in other cases of programmed cell death, two genes, *bcl-2* and *bax,* are emerging as important players in this developmental process. It is currently believed that bcl-2 is a death-repressor protein, whereas bax is important in the cell-death pathway. Female mice deficient for *bcl-2* are fertile but have a marked reduction in the number of primordial follicles, compared with those in normal controls (Ratts et al. 1995). In addition, a number of primordial follicles are aberrantly formed or contain degenerating oocytes, although follicles that successfully enter the growth phase appear normal. The heterogeneity of the phenotype may reflect the complexity of interactions of multiple factors (e.g., gonadotropins, ovarian steroids, and growth factors) that operate in concert to determine the fate of the oocyte and follicle. In contrast to the *bcl-2*-mutant mice, *bax*-deficient mice are endowed with an increased number of primordial follicles, compared with those in normal controls (Tilly 1996), suggesting that bax and bcl-2 have antagonistic roles. The balance between germ-cell survival and apoptosis plays an important role in reproductive capacity, by determining the number of oocytes that are available during the reproductive years. A more rapid loss of oocytes from an environment favoring apoptosis may result in diminished ovarian reserve and may present, clinically, with infertility and an early age at menopause.

Gonadotropin-Independent Growth

After birth, the initial stages of folliculogenesis are gonadotropin independent and are thought to involve intraovarian growth factors (Kol and Adashi 1995). Growth-differentiation factor-9 (GDF-9), a member of the transforming growth-factor-β superfamily, is oocyte specific. GDF-9 mRNA is first expressed at the primary-follicle stage and persists even after ovulation. Female mice carrying a homozygous null mutation for *gdf9* are infertile (Dong et al. 1996). Their ovaries are small on gross anatomical examination, and follicular development does not progress past the primary-follicle stage. Some aspects of oogenesis, including growth and formation of a zona pellucida, proceed normally; however, organelle structures are missing or altered, and meiotic competence is impaired. These abnormalities may reflect arrested development of the surrounding somatic cells, reflecting the control that oocytes, by regulating their microenvironment, exert over their destiny. Gonadotropin levels are elevated in these mice, consistent with ovarian failure, and, thus, a deficiency in GDF-9 (or other oocyte-specific growth factors) may lead to premature ovarian failure.

Gonadotropin-Dependent Growth

The gonadotropins—follicle-stimulating hormone (FSH) and LH—are secreted from the anterior pituitary, in response to gonadotropin-releasing hormone (GnRH). Both the production of ovarian steroids and normal follicular maturation involve the coordinated ef-

forts of FSH and LH. The "two-cell hypothesis" of estrogen production proposes that LH stimulates theca cells at the periphery of the growing follicle, to produce androgens that are then converted (under the influence of FSH) to estrogens, in the granulosa cells closer to the oocyte. During the luteal-follicular transition, there is a rise in FSH, which is necessary for the recruitment of a cohort of antral follicles. In mice, multiple follicles grow and ovulate eggs, whereas, in primates, usually one follicle achieves dominance and proceeds to ovulation while the others become atretic. Although this selection process is incompletely understood, FSH plays a central role by establishing an estrogenic microenvironment in the dominant follicle. As peripheral estradiol levels increase, FSH is lowered by a negative-feedback loop; and the less competent follicles cannot survive in a state of relative FSH deficiency.

Gonadotropins are heterodimeric hormones that share a common α subunit but have distinct β subunits. Because the release of FSH and LH are under similar control, it is difficult to distinguish the role of each independently of the other. Recently, an isolated FSH deficiency was created in mice by a targeted mutation in the β subunit of *fsh* (Kumar et al. 1997). Females homozygous for the mutation are infertile. Their small ovaries contain neither antral-stage follicles nor corpora lutea, indicating that these animals fail to ovulate. The infertile phenotype is reversed by exogenous gonadotropins and confirms the critical role of FSH beyond the secondary stage of follicular maturation. In humans, spontaneous mutations of FSH and its receptor have been reported. A 15-year-old female who is a compound heterozygote, carrying two mutations in exon 3 of the *FSH*β gene, displayed primary amenorrhea and delayed puberty (Layman et al. 1997). Unaffected family members were heterozygous and possessed one copy of the mutant allele as well as a normal allele. Similar clinical characteristics were reported in a woman with a homozygous frameshift mutation identical to one of the mutations found in the patient described above. This woman conceived after exogenous FSH was administered, demonstrating the same phenotype reversibility observed in the mouse model (Matthews et al. 1993).

The actions of FSH and LH are mediated by specific G-protein–coupled receptors. Although the LH receptors are found on both granulosa and thecal cells, the expression of FSH receptors is restricted to the granulosa cells. An inactivating mutation of the FSH receptor results in hypergonadotropic ovarian failure, and such a mutation may be responsible for a subgroup of women with normal karyotypes but premature ovarian failure. In a Finnish population, a recessively inherited, inactivating 566C→T mutation in the *FSH* receptor gene has been reported in six families and has been designated the "FSH resistant ovaries" (FSHRO) syndrome (Aittomaki et al. 1995). Most females with FSHRO are estrogen deficient and have hypoplastic ovaries with no antral follicles. Recently, an inactivating mutation in the LH receptor was reported in a young woman who presented with primary amenorrhea and infertility (Toledo et al. 1996). Ovarian histology revealed normal stages of follicular development but no corpora lutea or corpora albicans. This supports the notion that FSH is sufficient for follicular development but that LH is crucial for ovulation. Long-standing hypoestrogenism in this same individual, indicated by her small uterine size, thin vaginal walls, and reduced bone mass, is consistent with the two-cell hypothesis of estrogen production, in which LH is necessary for the production of androgens, which are then converted to estrogens in the granulosa cells, under the influence of FSH.

Ovulation and Fertilization

During most of folliculogenesis, the oocyte is held in meiotic arrest and does not complete the first meiotic division until just prior to ovulation. After ovulation, the residual follicle is transformed into a corpus luteum. Gap junctions formed between granulosa cells and oocytes have been implicated in the maintenance of meiotic arrest. Connexins, a class of proteins that directly connect adjacent cells, are an integral component of gap junctions, and connexin37 participates in the gap junctions formed between granulosa cells and the oocyte. Female mice carrying a homozygous mutation in the connexin37 gene (*cx37*) are infertile, and follicular development is arrested at the early antral stage (Simon et al. 1997). Although premature resumption of meiosis is not observed in mice lacking connexin37, premature luteinization is detected, which suggests that oocyte signals are important in the inhibition of these changes and in the control of the formation of the corpus luteum. This model supports the importance of two-way communication between the granulosa cells and the oocyte, in coordinating the process of folliculogenesis (Eppig et al. 1997). Many features of this phenotype are similar to those observed in women with a normal karyotype and premature ovarian failure.

Fertilization occurs in the ampulla of the oviduct, where acrosome-intact sperm pass through the enveloping cumulus oophorus (composed of a glycosaminoglycan matrix and cumulus cells) and contact the ovulated egg. The binding of sperm to the zona pellucida induces the acrosome reaction, a sudden release of stored lytic enzymes. These enzymes may modify the sperm surface proteins, or they may degrade the zona pellucida and allow sperm to reach the plasma membrane. After zona penetration, the acrosome-reacted sperm fuses with the egg's plasma membrane, enters the cytoplasm, and

forms the male pronucleus of the one-cell zygote. This sperm-egg fusion induces the cortical granules, located at the periphery of the egg, to fuse with the egg plasma membrane and to release their contents into the perivitelline space, modifying the zona so that it no longer permits subsequent sperm binding or penetration. Thus, the zona plays a key role in the relatively species-specific events of fertilization and the postfertilization block to polyspermy (Yanagimachi 1994).

Formation of the Zona Pellucida

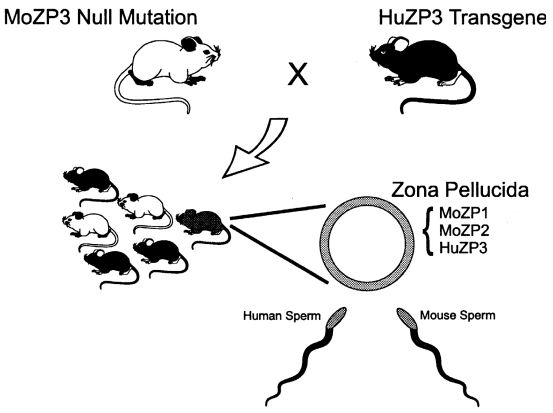
In mice and humans, the zona pellucida is composed of three sulfated glycoproteins (ZP1, ZP2, and ZP3), the primary amino acid sequences of which were initially deduced from cDNA. Of the three zona proteins, mouse and human ZP3 are the most conserved, each having polypeptide chains of 424 amino acids, 67% of which are identical (77% are similar). Mouse and human ZP2 contain 713 and 745 amino acids, respectively, 61% of which are identical, whereas mouse ZP1 (623 amino acids) contains 83 additional amino acids, compared with the human homologue (540 amino acids), and, when aligned, only 43% of the amino acids are identical. Each zona protein is posttranslationally modified, and the apparent masses of the glycoproteins that participate in the insoluble zona matrix are quite different between the two species.

The specificity of sperm binding to heterologous zonae pellucidae differs among mammals, and the role of individual zona proteins in the mediation of sperm binding in vitro has been variously described (see sidebar). Purified mouse ZP3 inhibits sperm binding to the zona pellucida and induces the sperm acrosome reaction, a prerequisite to zona penetration. Mouse ZP2 is proposed to be a secondary sperm receptor, binding to acrosome-reacted sperm, and a current model suggests that ZP1 plays a structural role by cross-linking filaments composed of ZP2/ZP3 heterodimers (Wassarman 1988). Although no direct evidence exists for human ZP3 acting as the primary sperm receptor, such a function has been inferred from its high degree of homology with mouse ZP3. In other species (i.e., pig and rabbit), homologues of ZP1 also have been implicated in the mediation of sperm binding to eggs; and data in a report indicate that gp69/64 (a homologue of ZP2) is responsible for sperm binding in *Xenopus laevis* (Tian et al. 1997).

Targeted disruption of individual zona genes in the mouse can provide additional insight into the roles of the zona pellucida in folliculogenesis, fertilization, and early development. For example, females that carry a null mutation of ZP3 possess no visible zonae pellucidae and are infertile (Liu et al. 1996; Rankin et al. 1996). Analyses of the ovaries from these mice indicate that

"Humanizing" the Mouse Egg Coat

The zona pellucida is a specialized extracellular matrix that surrounds each ovulated egg. It consists of three major glycoproteins that are unique to the zona and that are expressed in all mammals. Despite this simple structure, the roles of the zona are complex: it serves in the initial binding of sperm to the egg, it initiates the acrosome reaction in bound sperm, and it is modified rapidly after the gametes fuse, to prevent fertilization by more than one sperm. The molecular basis of each step remains a matter of active research (Snell and White 1996), but investigators have begun to tease them apart, using transgenesis. Female mice that carry an insertional mutation in the *Zp3* gene (moZp3 null) express ZP1 and ZP2 but not ZP3 (Liu et al. 1996; Rankin et al. 1996). Such animals fail to assemble a zona matrix and are infertile. Recently, we have created transgenic mouse lines (huZP3) that express human ZP3 in their growing oocyte (Rankin et al., in press). The human protein expressed in mouse eggs is posttranslationally modified and incorporated into the zona matrix as a glycoprotein that is similar to, if not identical to, native human ZP3.



Taking advantage of the observation that human sperm will not bind to mouse eggs (Bedford 1977), we have crossed huZP3 mice with moZp3 null mice to produce huZP3 strains, whose chimeric zonae pellucidae contain mouse ZP1 and ZP2 but human ZP3. Despite the presence of human ZP3 in these zonae, human sperm do not bind to huZP3 rescue eggs. Also, notwithstanding the absence of mouse ZP3, mouse sperm bind and fertilize the eggs. A simple genetic explanation of these results is that, if a single human zona protein is required for human sperm binding, it is probably not human ZP3. However, the specificity of sperm binding might be determined not by the protein but by either its posttranslational modifications (which would be consistent with prevailing paradigms for mouse fertilization) or by the arrangement of the zona proteins in some characteristic supramolecular structure. We have also created null mutations in the Zp1 and Zp2 genes, which should allow us to probe the function of the human homologues in a similar fashion. If more than one zona protein is required for sperm binding, simple crosses can produce mouse lines with zonae pellucidae containing combinations of two or, eventually, all three human zona proteins, to create a partially or fully "humanized" zona pellucida. These transgenic mouse lines may be useful not only in determining the molecular basis of human sperm binding but also as diagnostic reagents for investigating the causes of infertility in human couples. (Figure adapted from Castle and Dean 1996.)

early folliculogenesis proceeds normally but that, as follicles enter the antral phase, many are lost (J. Eppig and T. Rankin, unpublished observations). Those follicles that do progress to preovulatory stages contain disorganized cumulus-oocyte complexes, and, in extreme cases, oocytes are no longer associated with the cumulus cells in the preovulatory antrum (Rankin et al. 1996). However, ovulation does occur in these animals, although few eggs are retrieved from the oviducts. The combination of oocyte loss after the antral-follicular stage and the virtual absence of eggs in the oviducts after ovulation likely accounts for the infertility of these mice. Even if fertilized in vivo, zona-free embryos cannot pass down the oviduct, presumably because of adhesion to the lining epithelial cells. This striking phenotype of zona-free eggs has not been reported in humans, despite the fact that eggs from hundreds of thousands of infertile women have been retrieved since the first in vitro fertilization resulting in a live human birth (Steptoe and Edwards 1978).

Conclusion

Folliculogenesis is a highly orchestrated series of events that result in the ovulation of an egg ready for fertilization. The use of targeted mutagenesis has provided insights into normal follicular physiology, and the creation of mouse models for human disease has enhanced our understanding of these disorders. These animal models provide the means to test the safety and efficacy of various treatment options, including, potentially, gene therapy. One can envision that slowing or halting the rate of follicular depletion through genetic manipulation may improve the reproductive potential of women who are either at risk for premature ovarian failure or undergoing cancer treatment with cytotoxic agents. In addition, both the establishment of model systems through the use of targeted mutagenesis and other advances in molecular biology will continue to help decipher the link between genetics and many reproductive diseases that have eluded diagnosis.

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